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2. Patent application number *(The Patent Office will fill in this part)* — 0221686.9 — 19 SEP 2002

3. Full name, address and postcode of the or of each applicant *(underline all surnames)* — The University of Ulster
Coleraine
Co Londonderry
BT52 1SA, Northern Ireland

Patents ADP number *(if you know it)* — ~~7384134001~~ 07121866001

If the applicant is a corporate body, give the country/state of its incorporation — United Kingdom

4. Title of the invention — "Biomarker"

5. Name of your agent *(if you have one)* — Murgitroyd & Company

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)* — Scotland House
165-169 Scotland Street
Glasgow
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6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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11. I/We request the grant of a patent on the basis of this application.

Signature Murgitroyd & Company Date 19 Sept 2002

Murgitroyd & Company

12. Name and daytime telephone number of person to contact in the United Kingdom — Roisin McNally — 0141 307 8400

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"Biomarker"

The present invention relates to the use of a novel biomarker for the prediction, diagnosis and management of diabetes mellitus together with its complications. More particularly, the present invention provides methods for diagnosing and predicting the onset of diabetes, through the detection or measurement of glycated insulin in plasma and biological samples.

Formation of advanced glycation-end products (AGEs) plays an important role in the long-term metabolic consequences of diabetes, including the ophthalmic, renal and atherosclerotic vascular complications. Glycation has been shown also to interfere with normal cellular functions including the activities of various enzymes such as Cu-Zn superoxide dismutase.

Glycated insulin has been identified in the pancreas of normal and diabetic animal models [1]; the

process of glycation in the beta cell is rapid and occurs in a time- and concentration-dependent fashion [2]. Glycated insulin exhibits a reduced ability to regulate plasma glucose homeostasis *in vivo* and to stimulate adipose tissue lipogenesis or glucose uptake and oxidation by isolated diaphragm and abdominal muscle *in vitro* [2-6]. Studies in healthy human volunteers using the hyperinsulinaemic-euglycaemic glucose clamp technique suggest that glycated insulin may contribute to insulin resistance states in type 2 diabetes mellitus [3].

The site of glycation of human insulin has now been identified by electrospray tandem mass spectrometry as the N-terminal Phe^1 of the B-chain [7], enabling the development of a sensitive and specific radioimmunoassay to measure concentrations of glycated insulin (and proinsulin) and to identify glycated insulin in pancreatic islets using immunohistochemistry [8].

One of the major pathophysiological consequences of long term evaluation of plasma glucose in diabetes is an increase in the non-enxymatic glycation (glycosaltion) of proteins. Glycation of structural, such as lens crystallins, has long been known to contribute to the development of many of the complications of diabetes. Glycation can further disrupt normal cellular funtions, including, for example, the activities of enzymes.

Insulin is known to be glycated in the pancreatic beta cells under conditions of hyperglycaemia. This glycation results in an impairment of insulin action.

It has been surprisingly shown herein that levels of glycated insulin are found to be elevated during the early stages of diabetes. Further, and unexpectedly, levels of glycated insulin have been shown to be inversely correlated with the duration (or stage) of diabetes. This observation as to the level of glycated insulin does not reflect, and in fact contradicts, the increase in the presence of other glycated molecules associated with diabetes, such as for example glycated haemoglobin, levels of which are seen to increase in accordance with the progression of diabetes.

Further, the secretion of glycated insulin following the glycation of insulin in pancreatic beta cells under conditions of glycaemia can be used to evaluate the competency of the beta cells, with the secretory activity and competency of the beta cells being deranged in prediabetes and individuals with increased risk of developing diabetes. In this sense therefore, levels of glycated insulin can be used in the prediction of the onset of diabetes.

The present invention accordingly provides for the novel use of glycated insulin as a biomarker which can be used in both the prediction and onset of diabetes. The detection and measurement of glycated

insulin can further be used in the management of diabetes along with associated complications.

The present invention aims to provide a convenient and effect means of being able to predict, diagnose and manage diabetes and its adherent complications on the basis of using assays for glycated insulin or proinsulin as a convenient biomarker. Prediction of the onset of the disease permits early counselling and intervention. Early detection of the disease enables patient treatment and management at an early stage.

According to the present invention there is provided a biomarker for use in the prediction of the onset of diabetes, the biomarker being glycated insulin.

Preferably the glycated insulin is defined as the linkage of one or more molecules of glucose, metabolites or related reducing sugars in the form of glucitol adducts, Schiff base adducts or amadori products to the human insulin molecule, the precursor form proinsulin or fragments thereof.

A further aspect of the present invention provides a method of predicting the onset of diabetes, the method including the steps of;

- obtaining a plasma or other suitable biological sample
- screening the sample for the presence of glycated insulin by any appropriate means.

Preferably the presence of glycated insulin in the sample is detected by means of radioimmunoassay (RIA).

A yet further aspect of the present invention provides a biomarker for the diagnosis of diabetes, the biomarker being glycated insulin.

Preferably the glycated insulin is defined as the linkage of one or more molecules of glucose, metabolites or related reducing sugars in the form of glucitol adducts, Schiff base adducts or amadori products to the human insulin molecule, the precursor form proinsulin or fragments thereof.

A further still aspect of the present invention provides for diagnosing diabetes, the method including the steps of;

- obtaining a plasma or other suitable biological sample
- measuring the amount of glycated insulin present therein

Preferably the presence of glycated insulin in the sample is detected by means of radioimmunoassay (RIA).

A yet further aspect of the present invention provides for the use of glycated insulin in the prediction of the onset and diagnosis of diabetes.

The present invention will now be described by way of example only, with reference to the accompanying drawings, wherein:

Figure 1 shows glycated haemoglobin (HbA_{1c}), plasma glucose and glycated insulin concentrations of control subjects and diabetic patients exhibiting good, moderate or poor metabolic control. Characteristics and numbers in each group are given in Table 1. Values are mean ± SEM. ***$p<0.001$ compared with control subjects.

EXAMPLE 1

Subjects. Mid-morning blood samples were withdrawn from type 2 diabetic subjects (n=102) attending routine hospital review appointments. All subjects were controlled by diet alone or by oral hypoglycaemic agents. No subjects were receiving insulin. Type 2 diabetic subjects were divided into 3 groups depending upon their glycaemic control. Group 1 represented subjects under good glycaemic control with a HbA_{1c} < 7% (upper limit of non-diabetic range, 6.5%). Group 2 was comprised of type 2 diabetic subjects with moderate glycaemic control (HbA_{1c} 7-9 %) and Group 3 represented subjects with a HbA_{1c} > 9% (poor glycaemic control). Age- and sex-matched normal healthy individuals served as controls. This study was approved by the ethics

committee and carried out following informed consent from all subjects.

Biochemical analyses. A specific RIA for glycated insulin has been developed [8]. In brief, an N-terminally glycated synthetic insulin peptide, closely related to the amino-terminal sequence of the insulin B-chain (Phe-Val-Asn-Gln-His-Leu-Tyr-Lys) was used to raise specific antibodies in rabbits and guinea pigs. This peptide comprised the naturally occurring 1-6 sequence of insulin B-chain with a Tyr and Lys substituted at positions 7 and 8 respectively. For determination of glycated insulin, the insulin peptide was glycated under hyperglycaemic reducing conditions and iodinated using the solid phase iodogen method, generating a high specific activity mono-iodinated I^{125}-tyrosylated glycated peptide tracer. A glycated insulin antiserum was used to establish a dextran-coated charcoal RIA with a glycated human insulin standard curve in the presence of insulin free serum. The glycated insulin antibody cross-reacted 56% with glycated proinsulin but cross-reaction with non-glycated insulin, proinsulin and other pancreatic hormones was negligible. Serum insulin was determined using a routine radioimmunoassay with human insulin standard. Glucose concentrations were determined using the glucose oxidase method and HbA_{1c} was measured in whole blood by ion-exchange HPLC [9]. Serum creatinine was determined using a multilayered dry slide aminohydrolase technique.

Statistical analysis. Data are expressed as mean ± SEM. Significant differences between groups of data were assessed using the unpaired Student's *t* test and statistical significance was assumed if $p<0.05$.

Results

Figure 1 illustrates that glycated insulin concentrations of diabetic patients exhibiting good and moderate metabolic control are raised relative to HbA_{1c}, plasma glucose and healthy control subjects.

The characteristics of the 75 control subjects and 102 diabetic patients are summarised in Table 1. Control and diabetic groups were well matched for age and sex. Serum creatinine concentrations were similar, indicating freedom from renal disease. BMI of diabetic groups was increased compared with controls. Duration of diabetes increased progressively in good, moderate and poorly controlled groups. A total of 70 diabetic subjects were taking oral hypoglycaemic agents of whom 16 were taking metformin alone, 27 sulphonylurea alone and 22 on a combination of metformin and sulphonylurea. The remaining patients were treated with dietary restriction alone (31%) or a combination of other treatments (5%), which included acarbose. The distribution of patients in each group on these treatment regimens is given in Table 1.

Control subjects had mean glycated haemoglobin values of 5.7 ± 0.1% compared with 6.4 ± 0.1 ($p<0.001$), 7.9 ± 0.1 ($p<0.001$) and 10.4 ± 0.4% ($p<0.001$) for good, moderate and poorly controlled diabetic groups, respectively (Figure 1, graph A). Plasma glucose concentrations were similarly raised in the diabetic groups, with highest values observed in the poorly controlled group (Figure 1, graph B). A positive correlation between glycated haemoglobin and glucose concentration was evident in the combined groups ($r=0.322$; $p<0.01$).

As shown in Figure 1, graph C, plasma glycated insulin concentration of good and moderately controlled diabetic groups were increased 2.4-fold ($p<0.001$) and 2.2-fold ($p<0.001$) compared with control subjects. The glycated insulin concentrations of the poorly controlled group was not significantly increased. Glycated insulin concentrations were higher in the well controlled group with the lowest glucose concentration and the shortest duration of diabetes, clearly indicating its potential as an early marker of diabetes and its potential complications. Insulin concentrations measured in the control, good, moderate and poorly controlled groups were 148 ± 19, 191 ± 25, 225 ± 40 and 159 ± 40 pmol/l, respectively.

Discussion

A growing body of evidence supports the role of glucose toxicity as an important contributor to the

progressive impairment of beta cell function and insulin sensitivity. Glycation of proteins as a result of prolonged hyperglycaemia plays an integral role in multi-system microvascular end-organ disease and both the DCCT and UKPDS studies confirm the benefits of improved glycaemic control.

Accumulating evidence supports the role of glycation of insulin as a feature of beta cell dysfunction and insulin resistance in diabetes. Evidence includes the demonstration of elevated glycated insulin in the pancreas of various animal models of diabetes [1,8,10] and the time- and concentration-dependent glycation of insulin in both isolated mouse islets or clonal insulin secreting cell lines under hyperglycaemic conditions [1,2].

The impaired biological activity of glycated insulin using *in vitro* studies with isolated muscle preparations and *in vivo* studies using healthy human subjects using hyperinsulinaemic euglycaemic glucose clamps has previously been demonstrated [3-6]. Furthermore, we suspect that glycated insulin acts as an insulin receptor antagonist. Having identified the major site of glycation of insulin a novel, highly sensitive and specific radioimmunoassay has been developed to detect glycated insulin and proinsulin in plasma and biological tissues.

In the present study, we have examined circulating glycated insulin by radioimmunoassay in 102 type 2

diabetic patients, classified into good, moderate and poorly controlled groups, in comparison with 75 age- and sex-matched controls. The results readily indicate that glycated insulin circulates at increased concentrations in type 2 diabetic patients with elevations of 2.4-fold and 2.2-fold in the good and moderately controlled diabetic subgroups, respectively. It is clear from these observations that the concentration of insulin circulating in the glycated form in diabetes is significant compared with non-diabetic control subjects. The levels of glycated insulin measured correlated inversely with the degree of hyperglycaemia and most importantly the duration of diabetes. The latter observation indicated that elevation of glycated insulin/proinsulin may be an early feature of beta cell dysfunction and diabetes.

In keeping with a time and concentration dependent glycation process, increased concentrations of plasma glycated insulin might have been anticipated in the most poorly controlled diabetic subjects. However the results of the present study show relatively reduced concentrations of circulating plasma glycated insulin and insulin compared to the higher concentrations in the moderately and particularly good controlled diabetic groups. Thus whereas a significant correlation existed between plasma glucose and glycated haemoglobin, concentrations of glycated insulin were not closely linked to either of these parameters.

This indicates, that glycated insulin is an early marker of diabetes and its complications, being dependent not only on the glycaemic environment but also the ability of the beta cells to transport and metabolise glucose to reactive phosphorylated forms at the intracellular sites of (pro)insulin synthesis and storage [1].

Additionally, the secretory activity and competency of the beta cells together with metabolic clearance rate will also determine the rates of delivery and removal of glycated insulin from the circulation. It is notable that these various parameters are deranged in prediabetes and in individuals with increased risk of developing diabetes.

A typical pattern of loss of first phase glucose-induced insulin secretion followed by progressively impaired second-phase insulin secretion, glucose blindness, and disproportionate hyperproinsulinaemia with impaired basal or steady state insulin secretion has been well observed in type 2 diabetes. It is also now well established that increasing hyperglycaemia and associated hyperlipidaemia lead to loss of differentiation of beta cells with down-regulation of genes essential for glucose transport and metabolism in the beta cells as well as for proinsulin synthesis [34, 35]. This contributes to the progressive beta cell dysfunction and reliance on hypoglycaemic drug therapy as observed in the poorly controlled subgroup. We propose that beta cell competency is a determinant of the total plasma

concentration of glycated insulin in individual patients depending on the status of their current disease.

Overall these data indicate that glycated insulin/proinsulin provides a novel and potentially important biomarker with potential in the prediction, diagnosis, and management of diabetes together with its complications. In this regard glycated insulin is defined as the linkage of one or more molecules of glucose, metabolites or related reducing sugars in the form of glucitol adducts, Schiff base adducts or amadori products to the human insulin molecule, the precursor form proinsulin or fragments thereof.

Although the present invention has been particularly shown and described with reference to a particular example, it will be understood by those skilled in the art that various changes in the form and details may be made therein without departing from the scope of the present invention.

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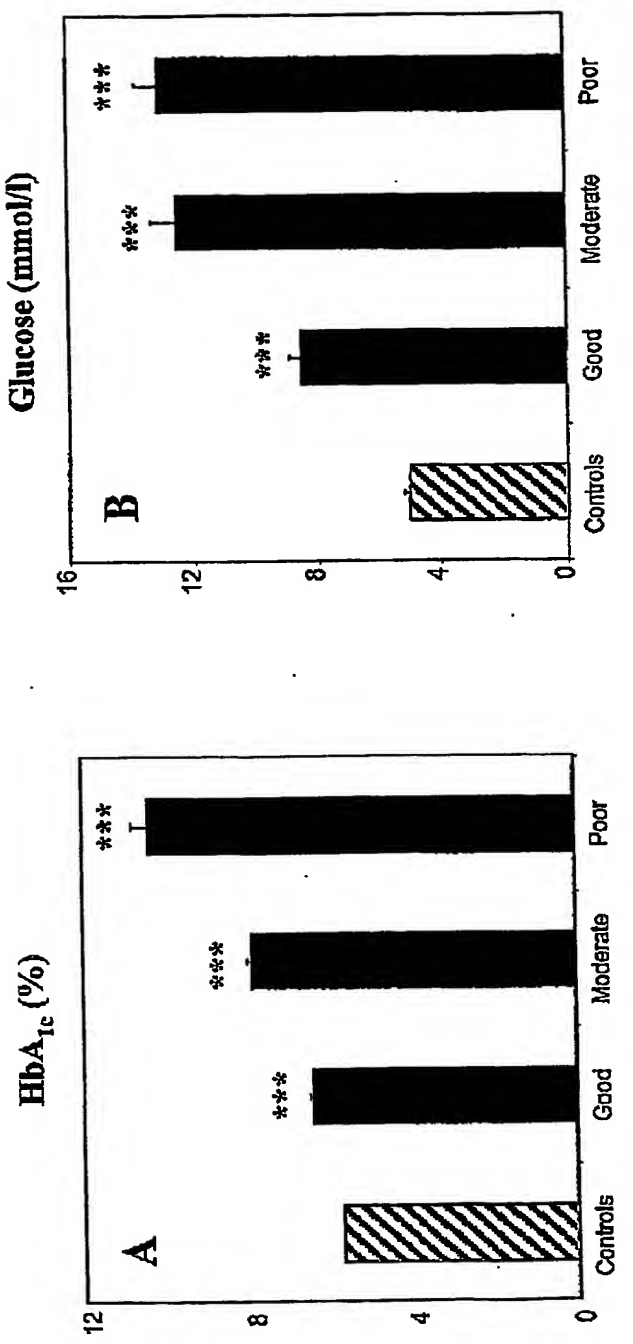
Table 1: Characteristics of controls subjects and type 2 diabetic patients.

	Control subjects	Patients with good control	Patients with moderate control	Patients with poor control
Number of subjects	75	44	41	17
Male/female ratio	31/44	21/23	24/17	9/8
Age (years)	59.5 ± 2.1	64.6 ± 1.4	63.4 ± 1.7	61.0 ± 3.7
Duration of diabetes (years)	--	4.8 ± 0.6	7.1 ± 0.9	8.5 ± 1.3
BMI (kg/m^2)	25.6 ± 0.5	29.6 ± 1.0***	30.6 ± 1.0***	28.9 ± 1.3**
Serum Creatinine (µmol/l)	86.0 ± 4.0	89.2 ± 3.6	89.0 ± 4.0	85.8 ± 3.6
Diet (%)	---	40.9	26.8	17.6
Metformin (%)	---	18.2	14.6	11.8
Sulphonylureas (%)	---	29.5	26.8	17.6
Metformin/ Sulphonyureas (%)	---	6.8	26.8	47.1
†Other drug combinations (%)	---	4.6	4.9	5.9

Values are Mean ± SEM. **$p<0.01$, ***$p<0.001$ compared with control subjects. †Other treatment combinations included metformin and acarbose, gliclazide and acarbose, and glibenclamide and acarbose.


A
HbA1c (%)
12
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Controls
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Glucose (mmol/l)
16
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Glycated insulin (pmol/l)
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Control
Good
Moderate
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Figure 1